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Several studies have confirmed that certain stress proteins can function as potent vaccines against a specific cancer when purified from the same tumor. We have hypothesized in our application that hsp110 may be an excellent candidate for cancer vaccines. In this second annual report, we describe the studies of the vaccine potential of hsp110 and its ER homologue grp170, two long recognized but unstudied stress proteins. Vaccination with these two HSPs purified from Meth A fibrosarcoma caused the complete tumor regression. A significant growth inhibition of colon26 tumor was also seen in the mice immunized with hsp110 or grp170 from Colon 26 tumor. In addition, HSP immunization significantly extended the life span of tumor-bearing mice when applied after tumor transplantation. A tumor specific CTL response developed in the mice immunized with tumor derived hsp110 or grp170. Furthermore, treatments of the mice with bone marrow derived dendritic cells pulsed with hsp110 or grp170 from tumor also elicited a strong anti-tumor response. Lastly, we showed that mild, fever-like hyperthermic conditions, which have been shown to stimulate other immunological functions, also stimulate the vaccine efficiency of hsp110 as well as hsc70, but not grp170. These studies indicate that hsp110 and grp170 can be used in heat shock protein-based cancer immunotherapy, that dendritic cells can be used to efficiently mediate this therapeutic approach, and that fever-level hyperthermia can significantly enhance the vaccine efficiency of heat shock proteins hsc70 and hsp110.

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## Table of Contents

## INTRODUCTION

Tumor derived heat shock protein (hsp)-peptide complexes (particularly hsp70 and grp94/gp96) have been demonstrated to serve as effective anti-tumor vaccines (1, 2). This approach takes advantage of the peptide binding properties of stress proteins which are responsible for their functions as molecular chaperones in numerous processes such as protein folding, transport, assembly, and peptide trafficking in antigen presentation (3, 4). Purification of specific heat shock proteins from tumor also provides its associated immunogenic peptides. In addition, vaccination with hsp/grp-peptide complexes derived from tumors circumvents the need to identify a large number of CTL epitopes of a cancer and the technical limitations associated with that approach.

Heat shock proteins (HSPs) are highly conserved and abundant proteins in both eukaryotes and prokaryotes. The heat shock proteins of mammalian cells can be classified into several families based on their size and structure (hsp25, hsp70, hsp90, and hsp110). However, in addition to hsps, a second set of stress proteins has been long observed which are localized in the endoplasmic reticulum (ER). These stress proteins have been referred to as glucose regulated proteins (grps) (such as grp78, grp94, and grp170), which are regulated by stresses which disrupt the function of the ER (5). While individual stress proteins have been studied for several years, the largest of the above hsp and grp groups, hsp110 and grp170, have been almost entirely ignored. These two stress proteins have only been cloned within the last few years and their characterization remains at a very preliminary level (6-12). In our laboratory, analysis of secondary structure indicated that, while exhibiting similarities to hsp70, hsp110 and grp170 appear to exhibit a peptide-binding cleft with significantly enlarged "lid" domain (13). This suggests that hsp110/grp170 binding affinities and/or capacities differ from hsp70. Our studies have confirmed that hsp110 exhibit a different peptide-binding capacity (9, 12) and grp170 is involved in immunoglobulin chain binding (14). Most notably, GRP170 may be the ATPase responsible for peptide import into the ER from TAP (Transporter associated with Antigen Processing) (15-17). Based on the previous studies on these two stress proteins in our laboratory and the previously demonstrated effectiveness of a few other stress proteins as vaccines, we hypothesized in our application that hsp110 may be an excellent candidate as anti-tumor vaccine. Objective of our application is to purify hsp110 from tumor cell and study its potential use in cancer immunotherapy. Here, in this second annual report, we describe the

analysis of the vaccine potential of hsp110. Furthermore, we extend our investigation to another high molecule weight heat shock protein grp170, which is an ER homologue of hsp110.

## BODY

*Purification of hsp110, grp170*

In addition to the further optimization of hsp110 purification from tumor tissue (Objective 1, task A), we also perfected the condition for isolating grp170 (ER homologue of hsp110). Briefly, A cell pellet or tissue was homogenized in hypotonic buffer by Dounce homogenization. The supernatant was further centrifuged at 100,000 g for 2 hours. Supernatant after contrifugation was applied to concanavalin A-sepharose column. The bound proteins containing grp170 were eluted with binding buffer containing 15% α-D-methylmannoside (Sigma). Con A-sepharose bound material was then applied on Mono Q column and eluted by 150~400 mM NaCl gradient. Pooled fractions were concentrated and applied on the Superose 12 column (Pharmacia). Hsp110 and grp170 were purified simultaneously from tumor and liver. The purity of the proteins was assessed by SDS-PAGE and silver staining as shown in figure 1. Approximately 20-50 μg hsp110 and 10-40 μg grp170 were obtained from each gram-wet weight of tumor or tissue. The yield of grp170 from tumor is usually higher than that from normal tissue as a result of a higher level of grp170 expression in the tumor, possibly due to a hypoxic tumor fraction.

While working out the purification protocol, we observed that hsp110 exists in a large complex of 400 kDa to700 kDa. Another two heat shock proteins hsc70 and hsp25 were found to directly interact with hsp110. Furthermore, tt was observed that luciferase migrate into this chaperone complex following heat shock, when it was added to this in vitro system, suggesting that these heat shock protein might form a chaperoning machine in vivo. Deletion mutant analysis demonstrates that peptide-binding domain is required for interaction with hsp25, but not with hsc70 (For further detail, see reprint attached in appendice).

*Vaccine studies of hsp110 and its ER homologue grp170*

As outlined in Objective 2 of application, we then investigated whether immunization with purified hsp110 and grp170 could protect mice against tumor challenge. For this purpose, the

methylcholanthrene-induced fibrosarcoma (Meth A) tumor model was initially employed. We immunized mice twice with 40 μg (dose based on preliminary data) hsp110 or grp170 and then challenged them with Meth A cells by intradermal injection as described in the Methods. Figure 2 shows the results of this study. Tumor growth data on individual animals are presented by separate lines, since some individual differences in the grp170 treated animals were observed. It is seen mice immunized with hsp110 and grp170 were protected from the Meth A tumor challenge. Interestingly, and similar to studies of others, most hsp110/grp170 vaccinated animals transiently developed tumors which then regressed and disappeared. However, in the mice which were immunized with grp170, two of five mice failed to develop any measurable tumor mass.

To test the generality of these observations on the vaccine activity of hsp110 and grp170 in the Meth A sarcoma tumor system, we next chose the Colon 26 tumor model. This model was chosen since it has proven to be less immunogenic than the Meth A and to be generally more resistant to various therapies. Groups of mice (five mice per group) were immunized with PBS or with varying quantities of tumor-derived hsp110 or grp170 in 200 μl PBS. These mice were then given booster injections one week later. Hsp110 or grp170 were also isolated from the livers of the same animals and this or PBS was used as control. Seven days after last immunization mice were injected subcutaneously on the right flank with Colon 26 tumor cells. As seen in figure 3, all mice that were immunized with PBS or liver derived hsp110 or grp170 developed rapidly growing tumors. In contrast, mice immunized with hsp110 and grp70 from Colon 26 tumor showed a significant tumor growth delay, in general agreement with the above Meth A results. The inhibitory of hsp110 or grp170 vaccination on Colon 26 tumor growth was dependent on the dose of hsp110 or grp170 used for immunization. While mice immunized with 20 μg (per injection) of hsp110 or grp170 only slightly slowed tumor growth, those immunized with 40 or 60 μg of hsp110 or grp170 showed increasingly significant tumor growth delays. Although tumor growth was not preventable in this highly aggressive and rapidly growing tumor system, this data demonstrates that hsp110 and grp170 have specific anti-tumor effects. On each day examined (e.g., 15, 21, 27 days after challenge), the mean volumes of the tumors that developed in mice immunized with hsp110 or grp170 at doses of 40 and 60 μg were significantly smaller than those of control mice ($p < 0.01$, student's t test). However, the differences in the mean volumes of the groups injected with PBS or liver derived hsp110/grp170 preparations were not significant. Lastly, mice immunized with Meth A derived

hsp110 or grp170 were not resistant to challenge with colon 26 tumor cells (data not shown). In these studies, it should be noted that although grp170 was purified using a Con A-sepharose column, contamination with Con A can be largely ruled out because the protective immunity was only be observed in mice immunized with tumor derived grp170 preparations and not normal liver preparations which also utilized Con A columns.

In considering the clinical application of a tumor vaccination strategy, it is more realistic to treat animals with tumor present at the time of vaccination. Thus, the aggressive Colon 26 tumor was again examined in using a therapy approach. Tumor cells (500,000) were transplanted into the flank of mice (10 mice each group). When tumors were readily palpable after inoculation, animals were treated with liver or Colon 26 derived hsp110 or grp170 on a weekly basis. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge. Tumor bearing mice treated with autologous hsp110 or grp170 preparations showed significantly longer survival times compared to the untreated mice or mice immunized with liver derived hsp110 or grp170. As shown in figure 3, all control mice died within 30 days, but approximately half of each group survived to 40 days and 20% of grp170 treated mice lived beyond 60 days, clearly demonstrating a beneficial anti-tumor effect. In parallel with the data shown in figure 2, this data suggests that grp170 is more efficient than hsp110 on an equal mass basis.

Since cellular immunity appeared to be critical in mediating the observed antitumor effects (18-20), we analyzed the ability of tumor-derived hsp110 and grp170 preparations to elicit a tumor specific $CD8^+$ T cell response. Mice were immunized twice, at weekly interval, with 40 μg of hsp110 or grp170 derived from Colon 26 or Meth A tumors. Splenocytes generated from these immunized mice were then cultured in vitro for 7 days with irradiated tumor cells. These cultured cells were then used as effector cells in CTL assay. As shown in Figure 5, a tumor-specific cytotoxicity was observed to occur against the tumor from which the immunogen (hsp110 or grp170) was derived. Splenocytes from mice immunized with Colon 26 cell derived hsp110 or grp170 preparations showed specific lysis for Colon 26 tumor cells only, but not Meth A tumor cells; conversely, splenocytes from animals immunized with Meth A tumor cells were only effective against Meth A cells and not Colon 26 cells. This demonstrates that vaccination with hsp110 or grp170 elicits a tumor specific CTL response. Splenocytes from naïve mice were unable to lyse both target cells

(control). Again, spleen cells derived from grp170 immunized animals yielded a greater % specific lysis than was obtained from hsp110 immunized animals.

Dendritic cells (DCs) have been known to be highly specialized antigen-presenting cells and to be the principal activators of naïve T cells in vitro and in vivo. Many have demonstrated that DC pulsed in vitro with tumor antigen, tumor extracts or mRNA are capable of stimulating specific CTL activity and protect animals against subsequent tumor challenge (21, 22). In order to investigate whether antigen presenting cells could be involved in the anti-tumor response elicited by hsp110 or grp170 immunization, we tested the ability of DCs to acquire an anti-tumor activity, presumably by presentation of hsp110 or grp170 chaperoned peptides. DCs were prepared from mouse bone marrow and then incubated with grp170 or hsp110 purified from the Colon 26 tumors for 3 hours at 37 °C. Cells were washed and resuspended in PBS. $10^6$ pulsed DCs in 100 μl PBS were used for intravenous injection for each mouse. The entire process was repeated 10 days later. Ten days after the second immunization, mice were challenged with $2\times10^4$ Colon 26 tumor cells and tumor growth was monitored by measuring the tumor diameter as shown in figure 6. It was observed that tumors grew rapidly in the mice that received PBS or (non-pulsed) DCs alone. However, tumor growth was significantly delayed in mice immunized with DCs pulsed with hsp110 or grp170. Grp170, once again, appeared to be more effective. Moreover, based on the immunization effects in the mice which received $10^6$ DCs pulsed with 20 μg protein and those that received two 40 μg protein by subcutaneous injections, it is found that hsp110/grp170 pulsed DC based immunotherapy was both more effective and used less protein.

Several recent studies have indicated that a modest increase in body temperature sustained for several hours, i.e. a condition comparable to common febrile response, can significantly affect certain immunological endpoints and immune function (23-26). We therefore exposed mice to 39.5 °C (i.e. core temperature) whole body hyperthermia (WBH) for a period of 8 hours to determine if hsp/grp vaccine efficiency might also be altered as a result of a fever-like thermal condition. Figure 7 compares the effectiveness of hsp110 and grp170, as well as hsc70 (40 μg each), derived from Colon 26 tumors taken from both normo-thermic (control) animals and animals previously exposed to this fever-like thermal treatment. This figure illustrates several points. First, hsc70 or hsp110 are significantly more efficient when purified from tumors derived from animals receiving prior fever-

range WBH. However, the prior fever-range thermal treatment is seen to reduce the vaccine efficiency of grp170. This data indicates that fever-like exposures can influence the antigen presentation pathway and/or peptide binding properties of these two (heat inducible) hsps purified from Colon 26 tumors but not a heat insensitive grp. In addition to these observations, this figure also shows that grp170 purified from unheated, control tumors (mice) is significantly more efficient in its vaccine efficiency when compared on an equal mass basis to either hsc70 or hsp110 (without heat). This increased efficiency of grp170 compared to hsp110 is also reflected in the studies described above. This comparison is based on administration of equal masses of these proteins and the enhanced efficiency of grp170 is further exacerbated when molecular size is taken into account (i.e. comparisons made on a molar basis). Third, hsc70 is seen here to be approximately equivalent in its vaccine efficiency (again, on an equal mass but not equal molar basis) to hsp110.

## KEY RESEARCH ACCOMPLISHMENTS

1. Further optimization of purification scheme for hsp110 and its ER homologue grp170.
2. Immunization of Meth A tumor derived hsp110 or grp170 cause the complete regression of tumor.
3. Vaccination with tumor hsp110 or grp170 results in significant tumor growth in colon26 tumor model. However, normal liver derived hsp110 or grp170 has no effect.
4. Hsp110 or grp170 immunization significantly improves the survivals of colon26 tumor-bearing mice.
5. HSP immunization elicits tumor specific CTL response.
6. Immunization with dentritic cells loaded with tumor derived hsp110 or grp170 induces anti-tumor response
7. Fever-like WBH enhances the vaccination efficiency of tumor-derived hsp110 or hsc70.
8. Based on the equal molar ratio, grp170 is more potent than hsp110 and hsc70 as anti-tumor vaccine.

## REPORTABLE OUTCOMES

**Publications:**

Wang, XY., Chen, X., Oh, Hyun-Ju., Repasky, EA., Kazim, L., Subjeck, J. (2000). Characterization of native interaction between hsp110 with hsp25 and hsc70 chaperones. FEBS Letter. 465(2-3): 98-102.

Wang, XY., Kaneko, Y., Repasky, E.A., Subjeck, J.R. (2000). Heat shock proteins and immunotherapy. Immunol Invest. 29(2): 131-137.

**Manuscript submitted:**

Wang, XY., Kazim, L., Repasky, EA., Subjeck, J. Characterization of hsp110 and grp170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity.

**Presentation:**

**1999** The 14th **International Conference on Immunology, Cancer Immunotherapy: Pitfalls/Solutions**. Buffalo, New York. Subjeck, J. R. Heat shock proteins and cancer immunotherapy (presentation).

**Abstract:**

**2000 Meeting on Molecular Chaperones & the Heat shock Response.** Cold Spring Harbor laboratory, Cold Spring Harbor, New York. Wang, XY., Repasky, E.A., Kazim, L., Manjili, MH., Li, Y., Subjeck, J.R. Antitumor immunity induced by the high molecular weight stress proteins.

**2000 DoD Breast Cancer Research Program, Era of Hope Meeting.** (by Department of Defense, U.S. Armay Medical Research and Materiel Command). Atlantic, Georgia, USA. Wang, XY., Kaneko, Y., Li, Y., Repasky, E.A., Kazim, L., Subjeck, J.R. Anti-tumor immunity elicited by two high-molecular-weight heat shock proteins.

## CONCLUSIONS:

- Tumor-derived high molecular weight stress protein, hsp110 or grp170 both stimulate tumor specific immunity. But HSPs from normal tissue do not induce anti-tumor response.
- Hsp vaccination induces tumor specific CTL response, indicating that induction of immunity with tumor-derived HSPs requires functional host CD8 cells.
- Antigen presenting cells (i.e. dentritic cells) are capable of representing HSP chaperoned peptides, suggesting that APCs are involved in HSP immunization elicited anti-tumor response.
- Hsp110 or grp170 from tumor cells can be used in heat shock protein based immunotherapy, Vaccination with Hsp-peptide complexes derived from tumor circumvents the need to identify a large number of CTL epitopes of cancers, because HSP chaperone and re-present all antigenic repertoire of cancer cells. Thus, tumor derived HSP could become a safe and reliable source of tumor-specific antigens for clinical application.

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## APPENDICES

Figure 1. Hsp110 and grp170 preparations from colon 26 tumor or liver of BALB/c mice. Hsp 110 (A) and grp170 (B) purified from colon26 tumor (lane 1, 3) and liver of BALB/c mice (lane 2, 4) were separated by SDS-PAGE, followed by silver staining (lane 1, 2) or immunoblotting analysis (lane 3, 4) using antibodies for hsp110 and grp170 respectively.

Figure 2. Immunization of mice with hsp110 or grp170 protects mice against Meth A tumor challenge. Mice were immunized subcutaneously with 40 μg of hsp110 or grp170 and boosted with the same amounts of these proteins 1 week later. 7 days after the second immunization the mice were challenged with 100,000 live Meth A tumor cells intradermally. Each group contained 5 mice and each line represents the kinetics of tumor growth in one mouse.

Figure 3. Immunogenicity of hsp110 and grp170 preparations purified from Colon 26 tumor. Mice were immunized twice with varying doses (20, 40 and 60 μg) of hsp110 and grp170 from Colon 26 tumor or liver of BALB/c mice as indicated. 1 week after the second immunization, mice were challenged with 20,000 live Colon 26 cells subcutaneously.

Figure 4. Effects of immunization with tumor derived hsp on the survival of tumor-bearing mice. Mice were first inoculated s.c. with 500,000 Colon 26 cells. After the tumor was palpable, mice were treated with or without 40 μg hsp110 or grp170 at weekly interval. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge.

Figure 5. Tumor specific CTL response elicited by immunization with tumor derived hsp110 or grp170. Mice were immunized twice PBS, hsp110 or grp170 (40 μg) at weekly intervals. 1 week after second immunization, splenocytes were isolated as effector cells and re-stimulated with irradiated Colon 26 or Meth A tumor cells in vitro for 7 days. The lymphocytes were analyzed for cytotoxic activity using $^{51}$Cr-labeled Colon 26 or Meth A cells as target cells.

Figure 6. Immunotherapy with DCs pulsed with hsp110 or grp170. DCs ($1\times10^7$) were generated from bone marrow of BALB/c mice and incubated with hsp110 or grp170 (200 μg/ml) in vitro for 3 hrs. DCs were washed with introduced to mice ($10^6$ cells in 100 μl PBS per mouse) by i.v. injection. The whole immunization process was repeated 10 days later. Mice were challenged with 20,000 Colon 26 cells 10 days after second immunization.

Figure 7. Fever-like WBH enhances the vaccination efficiency of tumor-derived hsp110 or hsc70. Mice were first inoculated subcutaneously with 100,000 Colon 26 tumor cells on the frank area. After the tumor reached a size of approximately 1/1 cm, WBH was carried out as described in Materials and Methods. Tumors were collected next day, and grp170, hsp110 and hsc70 were isolated. Mice were immunized twice at weekly intervals and then challenged with 20,000 live Colon 26 tumor cells.

Figure 1

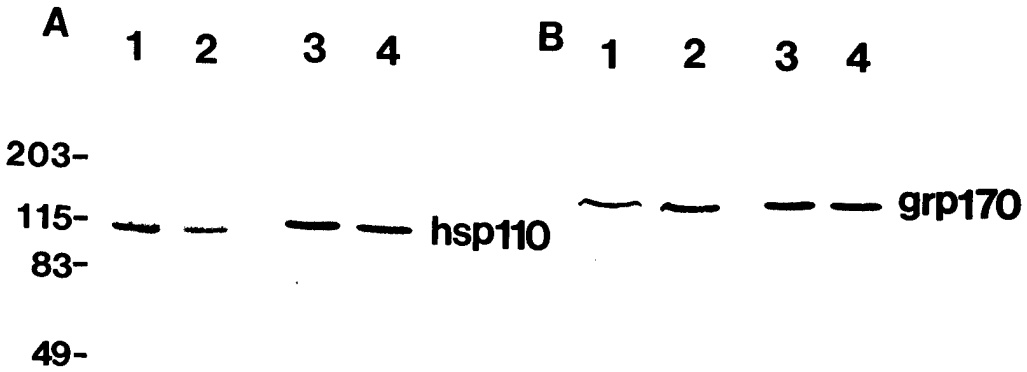
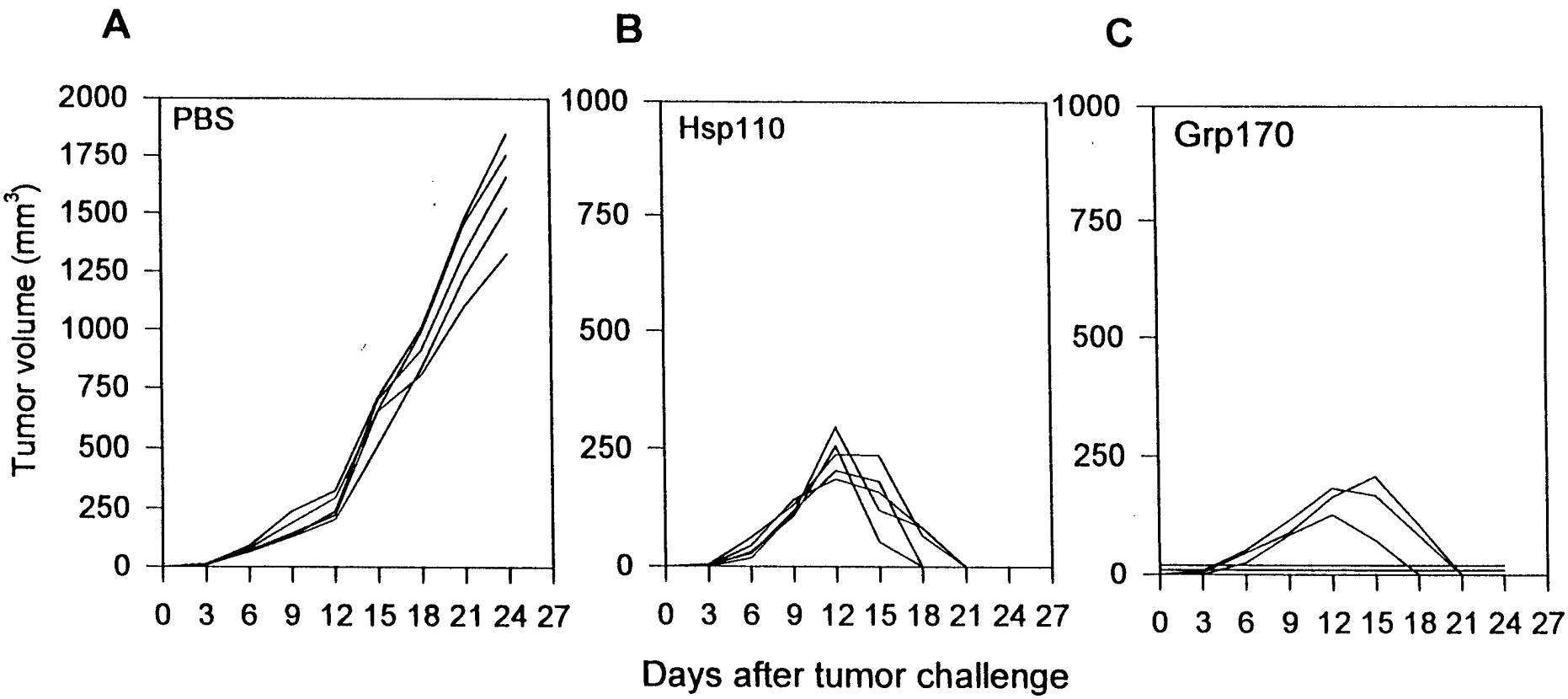


Figure 2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Days after tumor challenge

Figure 3

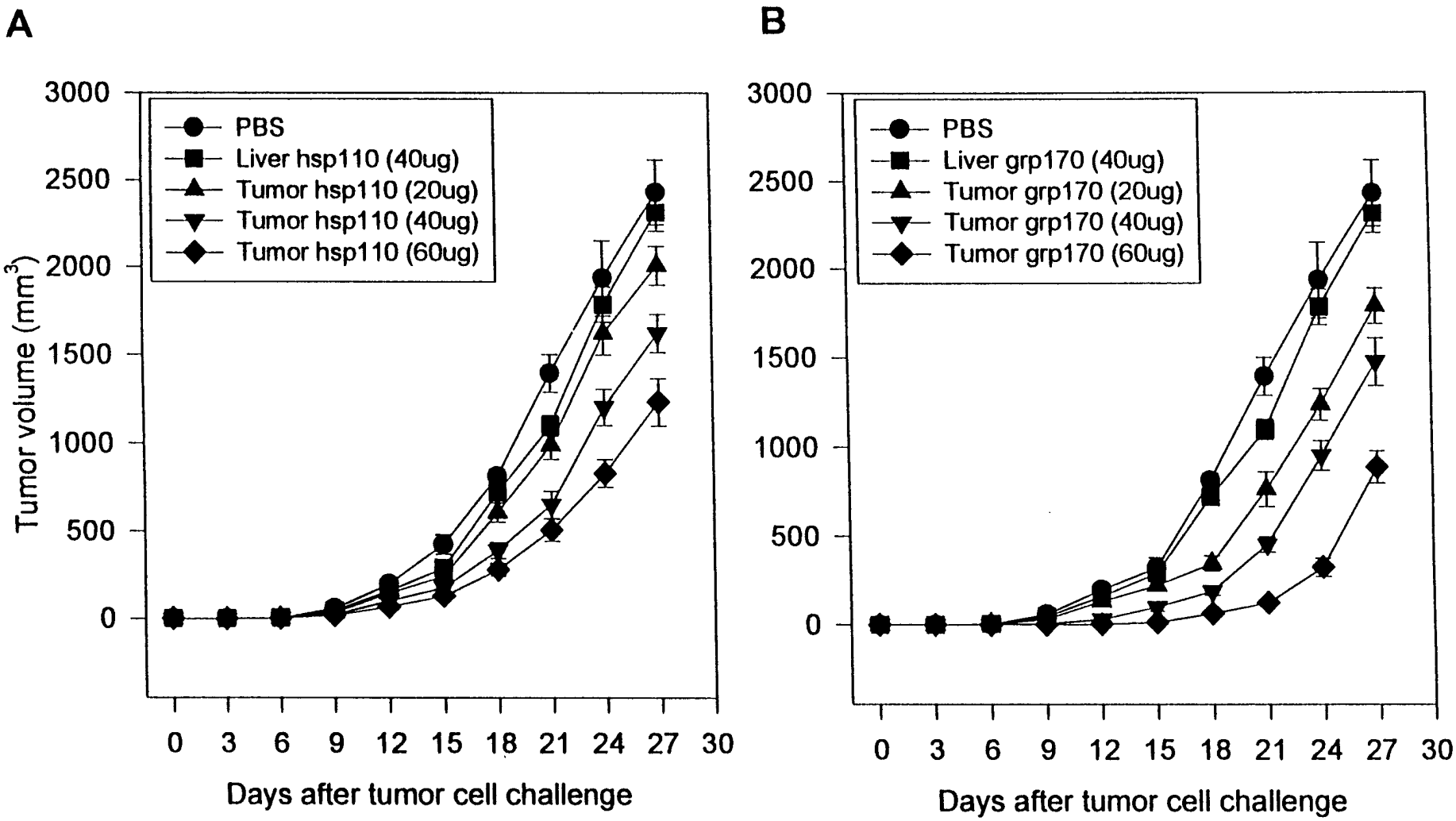
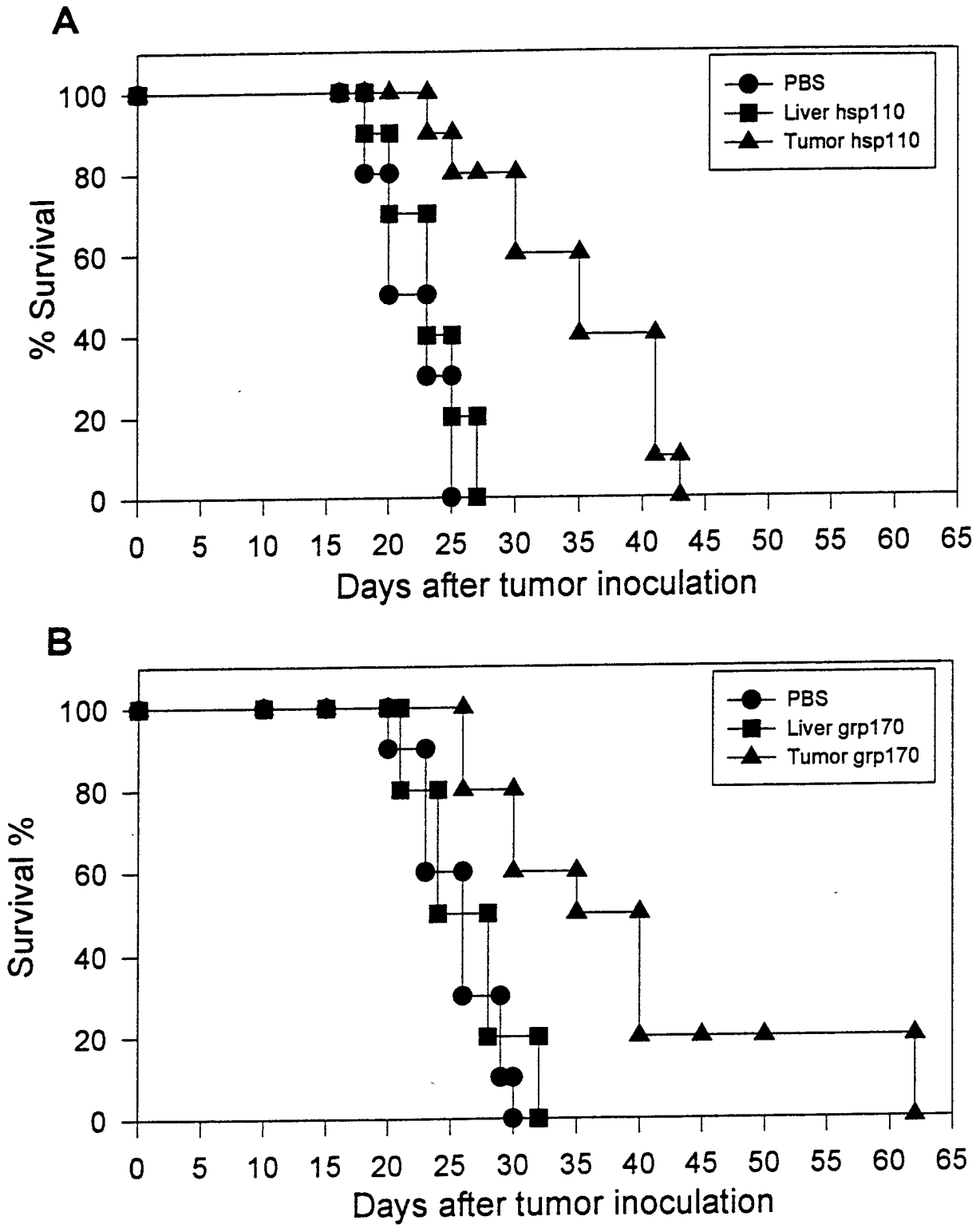

A
B
3000
2500
2000
1500
1000
500
0
Tumor volume (mm3)
PBS
Liver hsp110 (40ug)
Tumor hsp110 (20ug)
Tumor hsp110 (40ug)
Tumor hsp110 (60ug)
Liver grp170 (40ug)
Tumor grp170 (20ug)
Tumor grp170 (40ug)
Tumor grp170 (60ug)
0 3 6 9 12 15 18 21 24 27 30
Days after tumor cell challenge

Figure 4


A
PBS
Liver hsp110
Tumor hsp110
% Survival
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Days after tumor inoculation
B
PBS
Liver grp170
Tumor grp170
Survival %
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Days after tumor inoculation

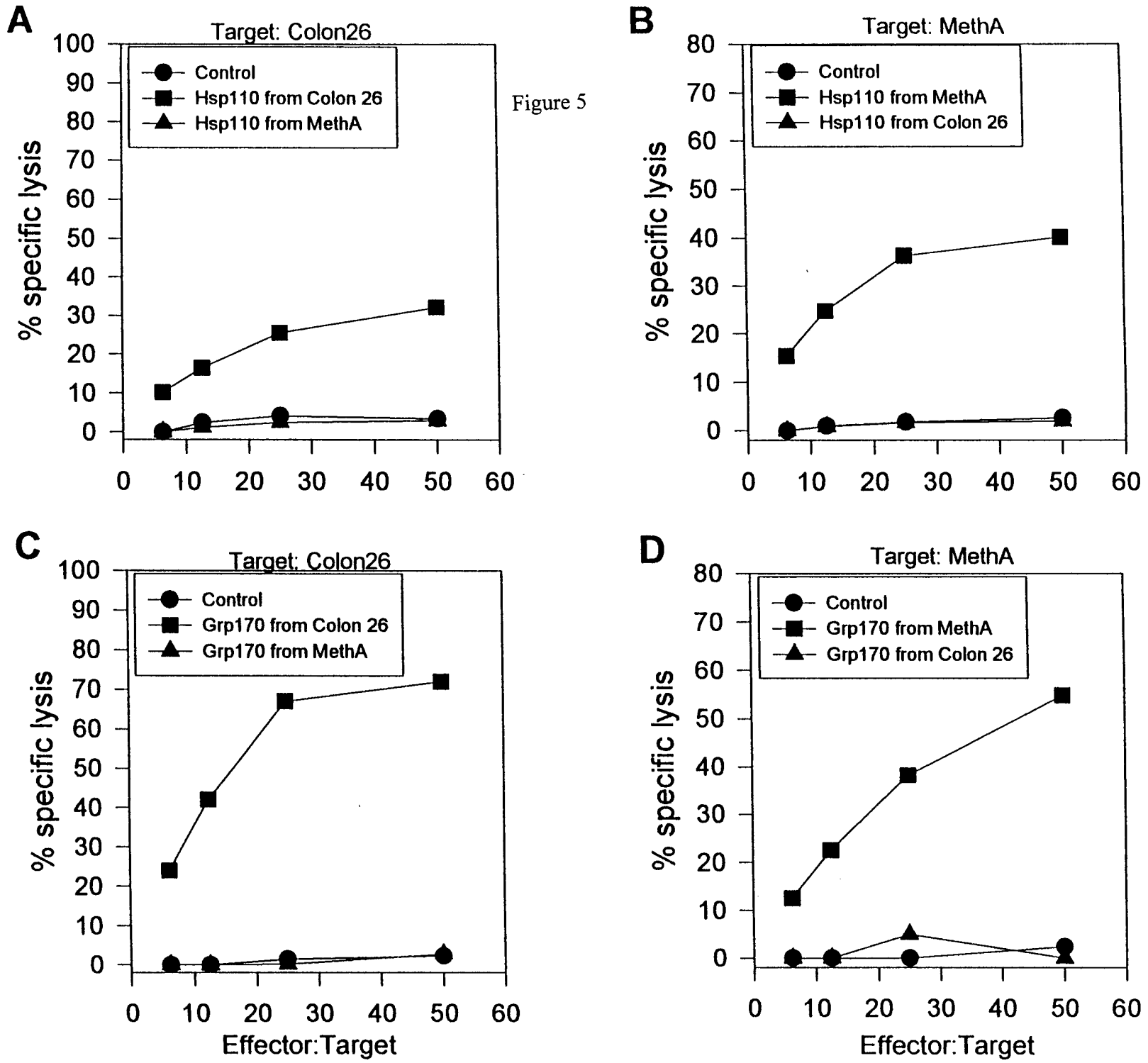
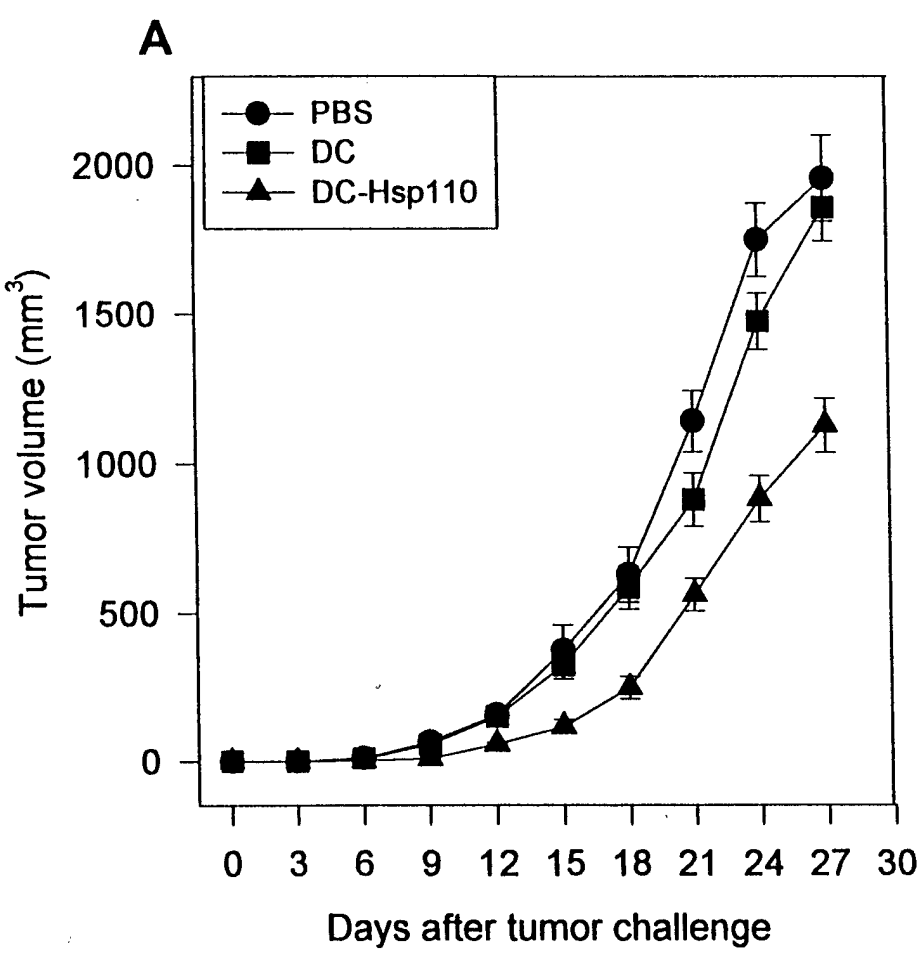

A
Target: Colon26
Control
Hsp110 from Colon 26
Hsp110 from MethA
% specific lysis
B
Target: MethA
Control
Hsp110 from MethA
Hsp110 from Colon 26
% specific lysis
C
Target: Colon26
Control
Grp170 from Colon 26
Grp170 from MethA
% specific lysis
Effector:Target
D
Target: MethA
Control
Grp170 from MethA
Grp170 from Colon 26
% specific lysis
Effector:Target

Figure 5

Figure 6


A
PBS
DC
DC-Hsp110
2000
1500
1000
500
0
Tumor volume (mm3)
0 3 6 9 12 15 18 21 24 27 30
Days after tumor challenge

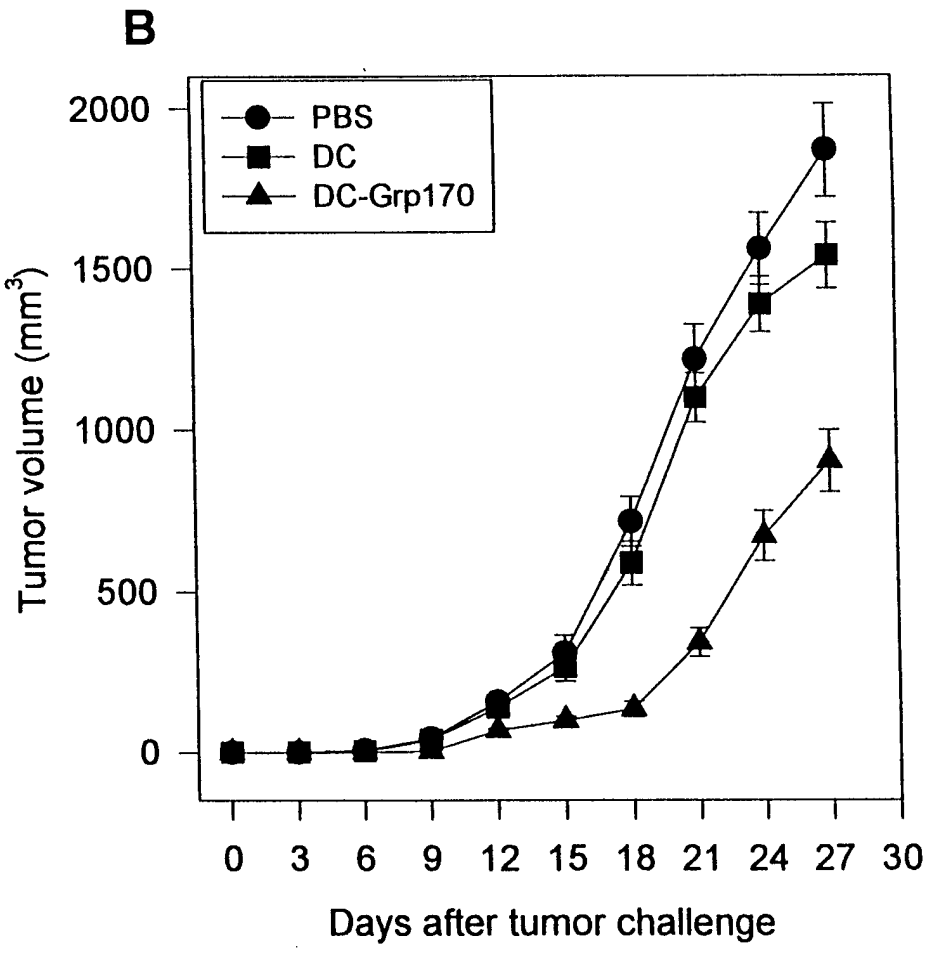
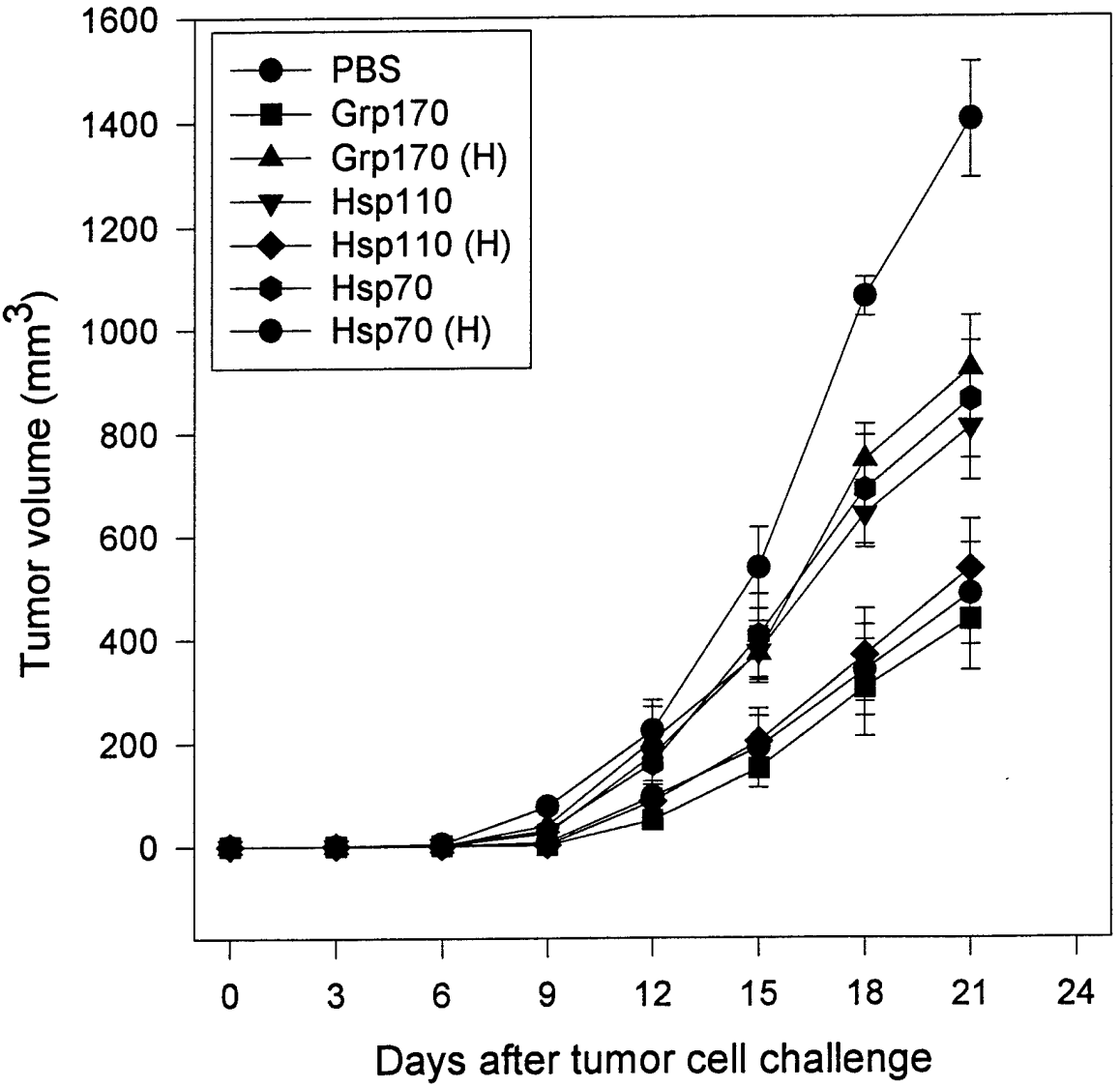

B
PBS
DC
DC-Grp170
2000
1500
1000
500
0
Tumor volume (mm3)
0 3 6 9 12 15 18 21 24 27 30
Days after tumor challenge

Figure 7


1600
1400
1200
1000
800
600
400
200
0
Tumor volume (mm3)
PBS
Grp170
Grp170 (H)
Hsp110
Hsp110 (H)
Hsp70
Hsp70 (H)
0
3
6
9
12
15
18
21
24
Days after tumor cell challenge

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# Characterization of native interaction of hsp110 with hsp25 and hsc70

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**Abstract The 110 kDa heat shock protein (HSP) (hsp110) has been shown to be a diverged subgroup of the hsp70 family and is one of the major HSPs in mammalian cells [1,2]. In examining the native interactions of hsp110, we observed that it is found to reside in a large molecular complex. Immunoblot analysis and co-immunoprecipitation studies identified two other HSPs as components of this complex, hsc70 and hsp25. When examined in vitro, purified hsp25, hsp70 and hsp110 were observed to spontaneously form a large complex and to directly interact with one another. When luciferase was added to this in vitro system, it was observed to migrate into this chaperone complex following heat shock. Examination of two deletion mutants of hsp110 demonstrated that its peptide-binding domain is required for interaction with hsp25, but not with hsc70. The potential function of the hsp110-hsc70-hsp25 complex is discussed.**

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*Key words:* Heat shock protein; Chaperone complex; Luciferase; Peptide-binding domain

## 1. Introduction

Heat shock proteins (HSPs) are a number of conserved proteins that can be induced in all organisms upon the exposure to stress conditions like high temperature. Many of them also function as molecular chaperones that prevent irreversible aggregation and assist protein folding or assembly. HSPs are divided into several major families based on their size and structure [3–6], the most well known being hsp110, hsp90, hsp70, hsp60/GroEL, hsp40/DnaJ and the small HSPs (sHSP, e.g. hsp25). All of these HSPs, except hsp110, have been extensively studied and their functions in cellular processes are broadly recognized today. Although the cloning of the hsp110 from hamster, mouse, yeast, *Arabidopsis*, fungi and a variety of other species has been recently described [7–14], the cellular functions of hsp110 are still not understood. Studies in our laboratory have been focusing on this large stress protein. We have previously shown that hsp110 is a significantly enlarged relative of hsp70 family, which also contains unique sequence elements not present in the hsp70s. We have also shown that hsp110 inhibits the aggregation of heat-denatured proteins in a highly efficient manner, sustains denatured proteins in a folding competent state, and confers thermotolerance when overexpressed in vivo [2]. In order to better understand this major stress protein in mammalian cells, its native interactions were investigated. We describe here studies indicating that hsp110 interacts in vivo and in vitro with two other major stress proteins, hsc70 and hsp25, and define the domains of hsp110 involved.

## 2. Materials and methods

### 2.1. Reagents

The rabbit anti-hsp110 antibody has been characterized previously [1]. Affinity-purified mouse anti-hsc70 monoclonal antibody, rabbit anti-murine hsp25 antibody, rat anti-hsp90 antibody and rat anti-TCP-1a monoclonal antibody, as well as recombinant hsc70 and murine hsp25, were all obtained from Stressgen Biotechnological Corp (Victoria, Canada). Anti-His Antibody was purchased from Amersham. Colon 26 tumor cells were cultured in DMEM supplemented with 10% calf serum in a 5% $CO_2$ incubator.

### 2.2. Plasmid construction and expression

Purification of recombinant His-tagged hsp110 and two deletion mutants used here has been described elsewhere [2,15]. Briefly, for the construction of hsp110 mutants, primers 5′-GCTAGAGGATCCTGTGCATTGCAGTGTGCAATT−/−CAGCGCAAGCTTACTAGTCCAGGTCCATATTGA-3′ (mutant #1, amino acids 375–858) and 5′-GACGACGGATCCTCTGTCGAGGCAGACATGGA−/−CAGCGCAAGCTTACTAGTCCAGGTCCATATTGA-3′ (mutant #2, amino acids 508–858) were used in the polymerase chain reaction (PCR). The PCR products were cloned into pRSETA vector (Invitrogen), and a $His_6$-(enterokinase recognition sequence) and additional Asp-Arg-Trp-Gly-Ser (for mutant #1) or Asp-Arg-Trp (for mutant #2) were added to the N-terminal of hsp110 mutants. Plasmids were transformed into *Escherichia coli* strain JM109 (DE3) and expression products were purified by $Ni_2$-nitrilotriacetic acid-agarose column (QIAGEN). The protein concentration was measured using the Bio-Rad protein assay kit.

### 2.3. Purification of native hsp110

Cells were washed with phosphate-buffered saline (PBS) and homogenized with a Teflon homogenizer with five volumes of buffer (30 mM $NaHCO_3$, pH 7.5, 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged for 20 min at 12 000×*g*, supernatants were further centrifuged for 2 h at 100 000×*g*. Cell extracts were first applied to Con-A-Sepharose column, unbound proteins were collected and loaded on ion exchange column (Mono Q, Pharmacia) equilibrated with 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.1 mM dithiothreitol (DTT). Bound proteins were eluted with a linear salt gradient (200 mM ~ 350 mM NaCl). hsp110 pooled fractions were concentrated using centricon 30 (Amicon) and applied to size exclusion column (Superose 6, Pharmacia) for high performance chromatography equilibrated with 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM DTT), then eluted with at a flow rate of 0.2 ml/min. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa) were used as protein markers.

### 2.4. Western blot analysis

Cells were washed with PBS and lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitors. After incubation on ice for 30 min, cell extracts were boiled with an equal volume of sodium dodecyl sulfate (SDS) sample buffer

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(50 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol) for 10 min and centrifuged at 10 000×*g* for 20 min. Equivalent protein samples were subjected to 7.5–10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto Immobilon-P membrane (Millipore, UK). Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, and then incubated for 2 h with primary antibodies diluted 1:1000 in TBST. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG diluted 1:2000 in TBST. Immunoreactivity was detected using the enhanced chemiluminescence detection system [16,17] (Amersham, Arlington Heights, IL, USA).

*2.5. Immunoprecipitation*

Immunoprecipitation was performed as previously described [18,19]. In brief, cells were washed three times with cold PBS and lysed in buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM ABESF, 0.025% $NaN_3$). The lysates were centrifuged and supernatant was presorbed with 0.05 volume pre-immune serum together with 30 ml protein A beads for 1 h. The lysates were incubated overnight at 4°C with hsp110 antibody (1:100) or hsc70 antibody (1:200) or hsp25 antibody (1:100). For in vitro analysis of interaction within chaperones, recombinant wild-type hsp110 and hsp110 mutants were first incubated with hsc70 or hsp25 at 30°C. Then, hsc70 antibody or hsp25 antibody were added and further incubated overnight at 4°C. Immune complex was precipitated with protein A-agrose (30 μl) for 2 h. Precipitates were washed three times with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 30–40 μl SDS sample buffer was added and boiled for 5 min. Supernatants were loaded to 7.5–12% SDS-PAGE and analyzed by immunoblotting.

*2.6. Interaction between luciferase and HSPs*

Luciferase (Boehringer Mannheim) was incubated with hsp110, hsc70 and hsp25 (150 nM each) in 25 mM HEPES, pH 7.9, 5 mM magnesium acetate, 50 mM KCl, 5 mM β-mercaptoethanol, and 1 mM ATP at room temperature or 43°C for 30 min. The solution was centrifuged at 16 000×*g* for 20 min, the supernatant was loaded on the Sephacryl S-300 column (Pharmacia) equilibrated with 20 mM Tris–HCl, pH 7.8, 150 mM NaCl and 2 mM DTT. The protein was eluted at a flow rate of 0.24 ml/min at 4°C. Fractions were collected and analyzed by Western blotting.

## 3. Results

*3.1. Existence of hsp110 as a large complex containing hsc70 and hsp25*

In order to investigate the physiological role of hsp110, our efforts have focused on the characterization of native hsp110 in Colon 26 cells. After cell extracts were applied to successive chromatography on Con-A-Sepharose and Mono Q columns, partially purified hsp110 fraction was loaded onto the Superose 6 size exclusion column (maximum resolution of 5000 kDa). It was observed that the Con-A and ion exchange-purified hsp110 fraction eluted from the Superose column in those fractions of size range between 200 and 700 kDa (Fig. 1A). Work was repeated using Sephacryl 300 (allyl dextran/bisacrylamide matrix) column and analysis provided similar data (data not shown).

Since hsp110 was eluted as one broad peak of high molecular mass, it is reasonable that this large in situ hsp110 complex might also contain additional components, potentially including other molecular chaperones and/or cellular substrates that may interact with hsp110. In order to investigate this possibility, we examined the purified hsp110 fraction derived from both ion exchange and size exclusion columns by immunoblotting for other HSPs using available antibodies. As shown in Fig. 1B, antibodies for hsp90, hsc70, T-complex polypeptide 1 (TCP-1) and hsp25 were used. All four proteins were readily detectable in the total cell lysate (lanes 1, 3, 5 and 7). When the hsp110 fraction was examined, TCP-1 and hsp90 were not observed (lanes 2 and 6). However, both hsc70 and hsp25 were found to co-purify with hsp110 with a significantly greater fraction of total cellular hsc70 present than of hsp25. Having found that hsc70 and hsp25 were also present, we determined their chromatographic profiles in the purified system (also shown in Fig. 1A).

To determine whether this co-purification also reflected an interaction between these three molecular chaperones, a recip-

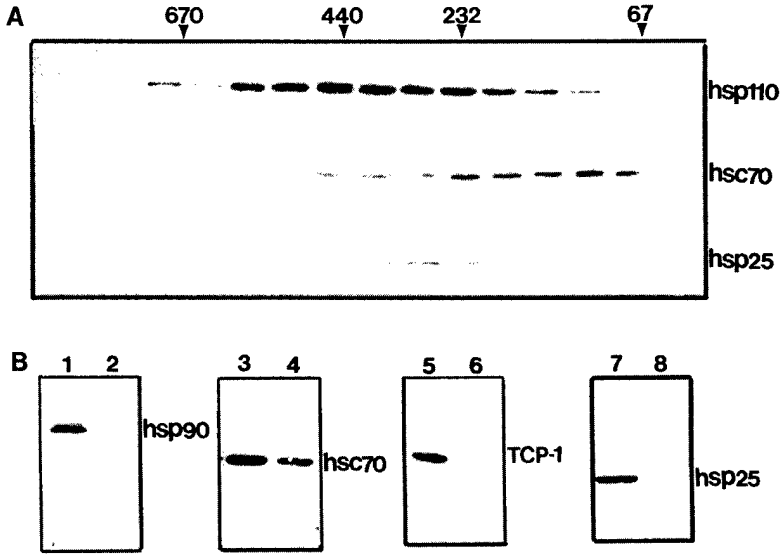


Fig. 1. Characterization of hsp110 complex. A: Chromatography profiles of native hsp110 separated by size exclusion column for FPLC. hsp110 was partially purified by successive chromatography on Con-A-Sepharose and Mono Q column. Pooled fraction was loaded on the Superose 6 column, proteins in each fraction were detected by immunoblotting with antibodies for hsp110, hsc70 and hsp25 (1:1000). B: Composition analysis of native hsp110 complex. Purified hsp110 fraction was detected by antibodies for hsp90 (lane 1, 2), hsc70 (lane 3, 4), TCP-1 (lane 5, 6) and hsp25 (lane 7, 8). Total cell extracts were also used as a positive control (lane 1, 3, 5 and 7).

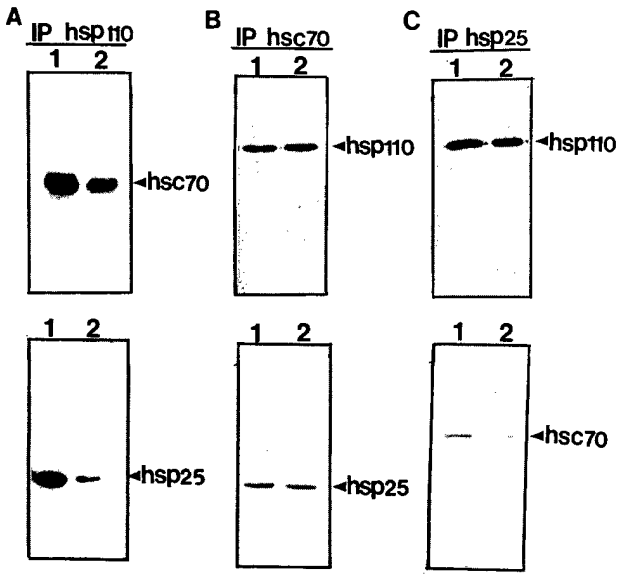


Fig. 2. Reciprocal immunoprecipitation between hsp110 and hsp70, hsp25. Cell lysates (lane 2) were incubated with antibodies for hsp110 (1:100) (A), hsp70 (1:200) (B) and hsp25 (1:100) (C), protein A-Sepharose was added and further incubated at 4°C overnight, immunoprecipitates were examined by immunoblotting with hsp110, hsp70 and hsp25 antibodies. Total cell extracts were also used as a positive control (lane 1).

rocal co-immunoprecipitation analysis was conducted with Colon 26 cell extracts and hsp110 fractions. Hsc70 and hsp25 were shown to precipitate with hsp110 using an anti-hsp110 antibody (Fig. 2A). Conversely, hsp110 was co-precipitated by an anti-hsc70 antibody or anti-hsp25 antibody (Fig. 2B,C, top). Pre-immune serum was also used to perform immunoprecipitation as a negative control with a correspondingly negative outcome (data not shown). Finally, interaction between hsc70 and hsp25 was analyzed by using antibodies for hsc70 and hsp25. Again, these two proteins were observed to co-immunoprecipitate with one (Fig. 2B,C, bottom). From the above study, we can conclude that hsp110, hsc70 and hsp25 interact in situ, either directly or indirectly.

### 3.2. *Analysis of interaction of hsp110 with hsc70 and hsp25 in vitro*

Next, we wished to determine whether hsp110, hsc70 and hsp25 interacted in vitro and whether they also were capable of forming a large molecular weight complex by using purified protein components. We then added luciferase as a potential substrate to this mixture since we have previously shown that hsp110 can solublize this reporter protein following heat denaturation. Luciferase, with hsp110, hsc70 and hsp25 mix (at 1:1 molar ratio) were incubated at room temperature or at 43°C for 30 min. The soluble fractions were loaded onto a Sephacryl S-300 column, eluted fractions were run on SDS-PAGE and analyzed by immunoblotting with antibodies for hsp110, hsc70, hsp25 and luciferase. The results of this study are presented in Fig. 3. It was found that hsp110, hsc70 and hsp25 are again present in high molecule weight fractions, however, these fractions were eluted at a significantly larger molecular size than that seen in vivo (Fig. 3A). Moreover, it was seen that heat treatment does not change the elution pattern for hsp110, hsc70 or hsp25. However, luciferase, which does not co-elute with the hsp110 complex prior to heating (being present as a monomer), was observed to move into a high molecule weight structure after the heat

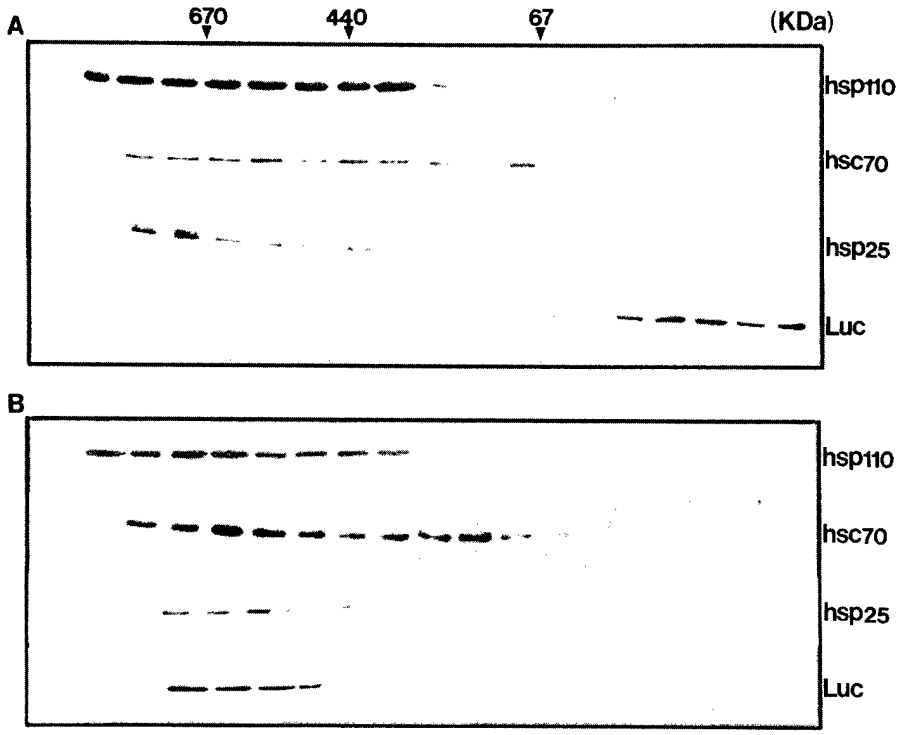


Fig. 3. Interaction between luciferase and HSPs complex. Luciferase and HSPs were incubated at room temperature (A) or 43°C (B) for 30 min and soluble fraction after centrifugation at $16000 \times g$ was loaded on Sephacryl S-300 column. The eluted fractions were analyzed by immunoblotting with antibodies for HSPs and luciferase.

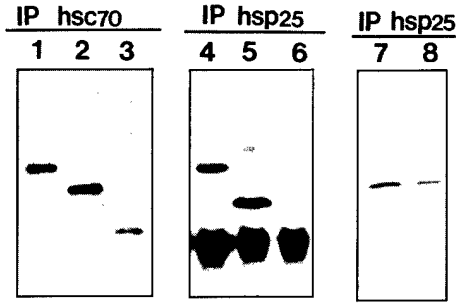


Fig. 4. Interaction analysis of hsp110 mutants and hsp70, hsp25 in vitro. *E. coli* expressed full-length hsp110 (lane 1, 4) and mutant #1 (lane 2, 5), mutant #2 (lane 3, 6) were incubated with hsc70 or hsp25 at 30°C for 1 h, then anti-hsc70 or anti-hsp25 antibodies were added. Immunoprecipitates were detected by anti-His antibody. In vitro interaction between hsc70 and hsp25 was also analyzed by the same method described above, hsc70 antibodies were used to test immunoprecipitate (lane 8). Total cell lysate was used as a positive control (lane 7). Equal amounts of protein (2 μg) for wild-type hsp110, hsp110 mutants, hsc70 and hsp25 were included in each assay.

exposure (Fig. 3B). Almost all of the luciferase was sustained in a soluble form in these experiments. When heated alone, luciferase became rapidly insoluble ([2] and data not shown). Heat shock did not affect the solubility of the three hsp110, hsc70 or hsp25.

The above data indicate that hsp110, hsc70 and hsp25 co-purify in a large molecular weight structure in vitro, as does luciferase (if present) after heating. This does not indicate how these proteins interact with themselves or that any two of them interact at all, although, that heated luciferase remains soluble is evidence for its interaction with at least one of the chaperones. Specifically, do both hsp110 and hsp25 bind to hsc70 but not to one another or can hsp110 and hsp25 interact on their own, etc.? To determine how these proteins interact, we again performed co-immunoprecipitation experiments using the pairs of purified proteins. Hsc70 and hsp110 were found to interact in the absence of hsp25 (Fig. 4, lane 1) and correspondingly hsp110 was observed to precipitate with hsp25 alone, in the absence of hsc70 (lane 4). Lastly, hsc70 and hsp25 also co-precipitate in the absence of hsp110 (lane 8).

Finally, we extended this in vitro study defining the interactions between hsp110, hsc70 and hsp25 by examining two deletion mutants of hsp110 which have previously been shown to represent the most simplistic (i.e. functional and non-functional) forms of this chaperone [15]. The first mutant examined (#1) lacks the N-terminal ATP-binding domain of hsp110, but contains the remaining sequence: i.e. the adjacent β sheet peptide-binding domain and other C-terminal sequences (size: 75 kDa and containing amino acids 375–858). This mutant has been shown to be fully functional in its ability to stabilize heat-denatured luciferase in a folding competent state. The second mutant used here (#2) again lacked the ATP-binding domain as well as the adjacent β sheet (peptide-binding) domain, but contained the remaining C-terminal sequence (size: 62 kDa and containing amino acids 508–858). This mutant has recently been shown to be incapable of performing the chaperoning function of sustaining heat-denatured luciferase in a soluble state. Mutant #1 (no ATP-binding domain) was observed to co-precipitate with both hsp70 (lane 2) and hsp25 (lane 5), indicating that these interactions do not involve its ATP-binding domain. However, mutant #2 (lacking both the ATP region and the peptide-binding region of hsp110) was observed to only associate with hsp70 (lane 3). This indicates that hsp25 and hsp70 can interact with hsp110 at different sites and that the association of hsp110 with hsp25 requires the peptide-binding domain of hsp110.

## 4. Discussion

The present study describes investigations into the native interactions of hsp110 in Colon 26 cells. We have found that hsp110 co-purifies with both hsc70 and hsp25 and further, that the three proteins can be co-immunoprecipitated. To determine that the co-immunoprecipitation results can reflect direct interactions between these chaperones and to also define these interactions, in vitro studies using purified hsp110, hsc70 and hsp25 were undertaken. It was found that these three chaperones also spontaneously form a large molecular complex in vitro. Moreover, this complex forms in the absence of an added substrate, but substrate (luciferase) can be induced to migrate into the complex by a heat stress. It is also shown that each pair of these proteins can interact directly, i.e. hsc70 with hsp110, hsc70 with hsp25, and hsp110 with hsp25. This, together with the co-precipitation data obtained from cell lysates, strongly argues that these interactions naturally occur in situ. Moreover, use of two deletion mutants of hsp110 demonstrates that its peptide-binding domain is required for hsp25-binding, but not for hsc70-binding and that its ATP-binding domain is not required for the interaction with either hsc70 or hsp25. This suggests that hsp110 may bind to hsp25 through its peptide-binding domain. That hsc70-hsp110-binding occurs in the absence of the hsp110 peptide-binding domain suggests that hsc70 may be actively binding to hsp110 through its (i.e. hsc70's) peptide-binding domain, but does not exclude the possibility that the two proteins interact via the involvement of other C-terminal domains.

These interactions between hsp110 and hsc70 raise questions as to how these proteins may function cooperatively. Since the peptide-binding domain of hsc70 and hsp110 appears to represent the 'business end' of these chaperones in performing chaperoning functions, it would be anticipated that their peptide-binding domains would be actively associated with substrate and not one another. This raises the possibility that this complex represents a chaperone 'storage compartment' which awaits cellular requirements. However, the migration of heat-denatured luciferase into this fraction following heat shock argues for an active chaperoning activity of the complex itself. It is possible that hsc70 may piggy-back hsp110 in a manner which allows transfer of substrate from hsp110 to hsc70 with subsequent folding in conjunction with DnaJ homologs and other chaperones. hsp110 has not yet been shown to have a folding function in conjunction with DnaJ co-chaperones, as is the case with hsc70 [2,15]. However, hsp110 exhibits different ATP-binding properties than do the hsp70s [15] and possible co-chaperones of hsp110 may be awaiting discovery. Previous in vitro studies have demonstrated that while sHSPs (e.g. hsp25) bind non-native protein [20–23], refolding still requires the presence of hsp70 [24]. Perhaps, hsp110 and sHSPs may act in the differential binding of a broad variety of substrates for subsequent shut-

tling to hsp70-DnaJ containing chaperone machines. Studies indicate, however, that an in vitro hsp110-hsc70-hsp25 complex (at a 1:1:1 molar ratio) is slightly less effective than is hsp110 alone in inhibiting luciferase aggregation following heat shock and further studies of this nature are necessary to determine or exclude a direct chaperoning function (Wang et al., data not shown).

That these three chaperones interact may represent a general phenomenon. Plesofsky-Vig and Brambl have recently shown that the small HSP of *Neurospora crassa*, called hsp30, binds to two cellular proteins, hsp70 and hsp88. Cloning and analysis of hsp88 have shown that it represents the hsp110 of *N. crassa* [25], suggesting that the interactions described here are phylogenetically conserved. In addition, Hatayama has described an interaction between hsp110 (referred to as hsp105) and hsp70 in FM3A cells [26]. The size of the hsp110 complex and the interaction with hsc70 observed in the present study (which also employed the added step of ion exchange chromatography) are clearly similar to, and in excellent agreement with this recent report. That hsp110 and hsc70 interact is also suggested by earlier studies from our laboratory which have demonstrated that hsp110 and hsc70 can interact and are functional in the folding heat-denatured luciferase [2]. Finally, it is noteworthy that hsp90 and TCP-1 were not observed in the hsp110 complex in the present study, despite its previously identified association with hsc70 and other proteins in the steroid hormone receptor [27–31]. However, it has recently been shown that SSE1 encoding a yeast member of the hsp110 family is required for the function of glucocorticoid receptor and physically associates with the hsp90 [32].

Several important questions are raised by the observations described here. For example, does this complex offer an enhanced capacity to hold a greater variety of substrate proteins in a folding competent state and/or to do so more efficiently, and is there an enhanced ability gained to refold denatured proteins in the presence of additional chaperones? Further studies are needed to define the function(s) of this hsp110-hsc70-hsp25 complex and how these chaperones interact with one another in the processing of substrate.

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# Heat Shock Proteins and Cancer Immunotherapy

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## ABSTRACT

Vaccination with heat shock proteins from tumor have been shown to elicit an anti-tumor response. Current studies indicate that the immunogenecity of HSPs is derived from the antigenic peptides which they associate with. Mechanisms by which the HSP-peptide complexes induce an immune response and the possible role of HSPs in antigen presentation is discussed in this article. The use of HSP-peptide complexes can be used as tumor vaccines for cancer immunotherapy is reviewed.

Heat shock proteins (HSPs) were first recognized as a set of polypeptides induced in *Drosphila* by elevated temperatures. They are highly conserved and abundant proteins in both eukaryotes and prokaryotes (1). Heat shock proteins are divided into several major families, Hsp110, 90, 70, 60/GroEL and the small HSPs based on their size and structure (2, 3, 4). HSPs have been found to be induced by environmental stress (e.g. heat shock, ethanol, heavy metal, glucose deprivation, inhibitor of glycosylation), pathological stress (viral infection, inflammation, fever and tissue trauma) (5) and even non-stressful conditions (cell cycle, cell differentiation and development) (6). Many HSPs also function as molecular chaperones that prevent irreversible aggregation and assist protein folding, unfolding, assembly and transport. These functions are based on the abilities of HSPs to bind unfolded peptide chains. The heat shock proteins are primarily localized in the cytoplasm and nucleus. A parallel set of stress proteins which are differentially inducible (e.g. by anoxia) are called GRPs and are localized in the endoplasmic reticulum. Primary GRPs fall into families parallel to the major HSPs: i.e. grp78, grp94(gp96) and grp170. Recently, HSPs and GRPs derived from tumors were shown to be able to protect mice against the subsequent challenge with tumor from which the HSPs were



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purified. This has been shown to be related to the general peptide binding properties of HSPs. Because of these findings, the role of stress proteins in tumor immunology has attracted significant attention.

**Tumor-derived HSPs elicit protective immunity against cancers**

In the early 1990's, Srivastava and his colleagues first found that a tumor rejection antigen, isolated by biochemical fractionation of tumor cells, was a heat shock protein (grp94/gp96) (7, 8, 9, 10). There is unequivocal evidence today that the HSPs, including hsp70, hsp90 and grp94/gp96, derived from a given cancer, can elicit protective immunity specific to that particular cancer. HSPs derived from normal tissues do not protect against any cancer tested. In the last several years, immunogenecity of HSP preparations from tumors has been repeatedly seen in different experimental tumor systems of distinct histological origins, which range from chemical or UV-radiation induced tumors to spontaneous tumors (11, 12, 13). Furthermore, two high-molecular-weight heat shock proteins, hsp110, grp170 derived from CT26 and MethA tumors (Wang et al., unpublished data) were recently found to induce an anti-tumor response against these tumors. Most importantly, it has been shown that the immunization of mice with gp96 resulted in the induction of memory T cells (14). Based on these observations, it is apparent that HSPs prepared from tumor are able to act as tumor vaccines.

**Immunogenicity of HSPs is due to their peptide-binding properties as chaperones**

Molecular cloning and sequence studies indicated that the genes coding for HSPs in the tumor cells and normal tissues did not exhibit any difference in nucleotide sequence (9). It was hypothesized that immunogenicity of HSPs lies not in the HSPs isolated from tumor or normal tissues, but rather in the peptides that they bind (8).

Studies of HSP structure and chaperoning properties by molecular biologists have provided significant evidence for peptide binding activities of heat shock proteins. It was shown that peptide-binding sites consisting of several β sheets exist in heat shock protein 70 (15). Furthermore, hsp70 association with peptides has been demonstrated in vitro (16, 17). Although the peptide-binding structure for gp96 is unknown, peptide-binding activities of gp96 have also been demonstrated. gp96 has been shown to transfer peptides from the transporter associated with antigen processing (TAP) to MHC class I molecules (18, 19, 20). Recently, TAP-independent peptides were also found to bind gp96 (21). In our laboratory, analysis of secondary structure indicated that, while exhibiting similarities to hsp70, hsp110 and grp170 appear to exhibit peptide-binding clefts with a significantly enlarged "lid" domain. This suggests that hsp110/grp170 binding affinities and/or capacities differ from hsp70. Recent studies have

confirmed that hsp110 exhibits a different peptide-binding capacity (22, 23). While little is known about GRP170 functions, it is evident that it is involved in binding immunoglobulin chain in the endoplasmic reticulum (ER) (24). Most notably, GRP170 may be the ATPase responsible for peptide import into the ER from TAP (18, 25, 26).

Recent studies from different laboratories also provide convincing evidence for the binding of antigenic peptide as the basis of the immunogenic activities of HSPs using a number of well-defined systems. Immunization with gp96 isolated from vesicular stomatitis virus (VSV) infected cells primed VSV-specific cytotoxic T lymphocytes (CTL) (27). Similarly, the gp96 isolated from β-galactosidase (β-gal) transfected cells elicited CTLs specific for β-gal and minor histocompatibility antigens expressed in these cells (28). This hypothesis is also directly supported by the observation that peptide-depleted hsp70 by ATP treatment was unable to elicit immunity against tumor challenge (29, 30). Most importantly, hsp70-peptide and gp96-peptide complexes can be reconstituted in vitro, and these complexes can induce peptide-specific CTLs (31). Young and colleagues also reported that immunization with recombinant hsp70-OVA fusion proteins protected mice against challenge with an OVA-expressing tumor (32). In contrast, fusion proteins not containing hsp70 were ineffective. All of these studies and others indicate that HSP-chaperoned peptides are responsible for the antigen specific immune response. Thus, HSPs have been suggested to be the first physiological mammalian adjuvant and they may be used as an antigen delivery vehicle for immunotherapy (31).

**Mechanism of HSP-peptides elicited immune response**

The mechanism through which immunization with HSP-peptide complex elicits antigen-specific CD8+ T cells is being worked out by a number of investigators. By using macrophage and T cell depletion studies, Udono et al demonstrated that this priming of the immune response by Hsp-peptide complex was sensitive to the functional abrogation of phagocytic cells (33). Macrophages were shown to internalize gp96-peptide complex and re-present the gp96-chaperoned peptide on the MHC I molecules (34). It was also found that peptide was actually recycled through a nonacidic compartment in the cell and not simply transferred to MHC class I molecule on the cell surface directly. Most interestingly, it seems that HSP-chaperoned peptides are independent of the MHC-type of the tumor from which they are derived, whereas, their presentation to the CTL is defined by the MHC phenotype of the APCs (34). Recently, it has been shown that immunization with bone morrow generated dendritic cells which were pulsed with tumor-derived HSPs elicited an anti-tumor response, suggesting that antigen presenting cells (APC) are critical for HSP-peptide complex mediated immune responses (Wang et al unpublished data). Furthermore, hsp70 released from tumor cells was seen to be internalized

directly into DCs and enhanced the capability of DCs to take up proteins/peptides, indicating that in addition to the function of chaperoning antigenic peptides from tumor, heat shock proteins themselves might act as a messenger to deliver an immunological signal to the host system (35). However the details of the intercellular events involved in the transfer of HSP-chaperoned peptides onto MHC class I molecules remains unknown.

Suto and Srivastava demonstrated that brefeldin A inhibited the presentation of HSP-chaperoned peptides (34). This study suggested that transport between endoplasmic reticulum (ER) and Golgi apparatus is necessary for the antigen re-presentation pathway. KDEL receptors recycle between Golgi and ER, thereby retrieving resident ER proteins that escaped from the ER (36). Also exogenous toxins such as Psedomonoas exotoxin and ricin are transported from Golgi to ER by interacting with KDEL receptors (37, 38). Thus, whether or not exogenous HSPs, especially ER resident GRPs, require KDEL receptors for their retrograde transport is an interesting possibility. In addition, the high efficiency of small quantities of HSPs to elicit an immune response indicates that there may exist HSP receptors on the surface of antigen presenting cells which are capable of taking up HSP-peptide complexes specifically. Arnold-Schild et al provided supporting evidence showing that gp96 and hsp70 bind specifically to the surface of APCs and are internalized spontaneously by receptor-mediated endocytosis. Furthermore, internalized HSPs were observed to co-localize with surface MHC class I molecules in early and late endosomal structures, indicating that HSPs are involved in the processes of antigen presentation (39) The HSP receptor may take up the HSP-peptide complex in a manner similar to that used for antigen uptake by the mannose receptor or the FC$\gamma$ receptor, which are also expressed on dendritic cells (40, 41, 42). Whether the uptake of HSPs is a specific or non-specific process still requires further study. Identification of the receptors responsible for the internalization of HSPs would contribute significantly to our understanding of the mechanism of the HSP-peptide complex elicited immune response.

**Potential of using HSP as tumor vaccines**

Each cancer has a specific antigenic fingerprint which consists of a large repertoire of mutated or non-mutated peptides (43). HSP vaccines are unique because of their ability to chaperone and represent a broad antigenic repertoire of tumor cells. Thus, vaccination with HSPs isolated from tumor cells circumvents the need to identify specific tumor antigens, and hence extends the use of HSP-based immunotherapy to the majority of cancers where specific tumor antigens have not yet been characterized. Moreover, since HSP vaccines are directed against the entire antigenic repertoire of that tumor, this avoids the possibility of immunological escape. All these studies suggest a promising future for HSPs as cancer vaccines.

Many HSPs are believed to be located in intracellular compartments, but cytosolic HSPs were recently found to be present on the cell surface and to be involved in the anti-tumor response. These HSPs seem to function as a target structure which can be recognized by γδ T cells and NK cells (44, 45, 46, 47). More interestingly, it was reported that the recognition of hsp70 on the target cells can be blocked by anti-hsp70 antibodies, but not by anti-MHC class I antibodies or anti-NK antibodies (48, 49). The endoplasmic reticular HSP grp94/gp96 was also seen to localize on the cell surface of tumor cells (50) and exposure of gp96 to microphages resulted in the secretion of a low level of cytokines, regardless of the peptides which gp96 binds (34). These observations are consistent with the idea that HSPs might act as antigen-presenting molecules themselves and possibly HSPs are involved in both antigen-specific and antigen-nonspecific immune responses. However, the mechanisms of HSP surface expression and its roles in the immune response still require further investigation. It is also conceivable that HSP-based immunotherapy may not only promote T cell-dependent anti-tumor immunity but also directly induce NK cell activation in vitro. Indeed, HSPs may be involved in the interaction between adaptive and innate immune responses.

It has been known that heat shock proteins not only protect cells from heat, but also render cells resistant to cell death induced by oxidative stress, TNF, and chemotherapeutic drugs (51, 52, 53). All of these data suggest that HSP expression in the tumor could enhance tumorigenesis and limit the efficacy of cancer therapy (54, 55). In addition, although HSP expression was recognized as a prognostic value in certain tumors, the data are limited and the results are contradictory (56, 57). Consistent with the observations that immunization with tumor-derived HSPs elicited tumor-specific immunity, it has been shown that immunogenicity of tumor cells co-segregate with the expression of heat shock proteins (58). Stable transfection of autologous HSP70 in tumor cells significantly enhances the immunogenicity of tumor, suggesting that increased levels of HSP may provide an immunostimulatory signal in vivo which helps break tolerance to tumor antigens (59). Based on the observations described above, heat shock proteins seem to play multiple functions in the tumor cell. It could increase the immunogenicity of tumor cells, while it could also help the tumor cell survive. Several questions arise: Is the high expression of HSP in the tumor a good or bad prognostic indication? What is the role of heat shock proteins in the tumor immunogenicity? Is it possible to manipulate tumor immunogenicity therapeutically if HSP expression correlates to immunogenicity of tumor? Do all HSP members perform similar immunological functions in the tumor? Can they all be used as tumor vaccines?

Collectively, the capability of HSPs to chaperone antigenic peptides and induce CTL has profound immunological implications. Although many questions remain unanswered, there is now unequivocal evidence from many laboratories that heat shock proteins (HSPs) can serve as

vaccines. Further studies of the physiological and immunological roles of HSPs in cells, including tumor cells, will help the translation of HSP-based immunotherpay into a new generation of anti-cancer vaccines against cancers.

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